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(54) Title: PROCESS FOR PRODUCING A MONOCYTE CHEMOTACTIC FACTOR POLYPEPTIDE AND A STRAIN FOR THE PRODUCTION THEREOF

(57) Abstract

A process for producing a monocyte chemotactic factor which comprises constructing an expression plasmid in which has been inserted DNA encoding a monocyte chemotactic factor consisting of 76 amino acids or a polypeptide which has truncated N-terminal amino acids thereof and having a terminator sequence inserted downstream of said DNA, introducing the expression plasmid into a selected *E. coli* such as an *E. coli* LE392 strain or the like to transform said *E. coli*, and cultivating the transformed *E. coli*.

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DESCRIPTION

Process for producing a monocyte chemotactic factor polypeptide and a strain for the production thereof

Background of the invention

Field of the Invention

The present invention relates to a process for producing a monocyte chemotactic factor polypeptide which is expected as a medicine for treating bacterial infectious diseases, improving immune functions, treating malignant tumor or the like.

Description of the Related Art

A monocyte chemotactic factor shows an activity to attract and activate the monocyte/macrophage which plays an important role in response to inflammation and immune reaction in defence mechanism of living bodies and is highly expected of clinical applications as a medicine for treating bacterial infectious diseases, improving immune functions, treating malignant tumors or the like.

It has been reported that a monocyte chemotactic factor was prepared from the culture supernatant of phytohemaglutinin (PHA)-stimulated human peripheral blood leukocytes or the like [Yoshimura, T. et al, J. Immunol., 142, 1956-1962, 1989; Robinson, E.A. et al, Proc. Natl. Acad. Sci. USA, 86, 1850-1854, 1989; Matsushima, K. et al, J. Exp. Med., 169, 1485-1490, 1989; Decock, B. et al, Biochem. Biophys. Res. Commun., 167, 904-909, 1990] and also prepared by recombinant DNA technology using DNA encoding a monocyte chemotactic factor with animal cells or E. coli as a host [Matsushima, K. et al. Cytokine, 1, 2-13, 1989]. Further, International Publication Number WO 90/07863 discloses a method of expressing a monocyte chemotactic factor as an unfused

polypeptide by recombinant DNA technology, namely a method of expressing said polypeptide by direct expression system, and also discloses that there was detected a monocyte chemotactic activity of said polypeptide.

However, a problem in the production of a monocyte chemotactic factor by these methods consists in that its mass production is so difficult that such a necessary amount of a monocyte chemotactic factor cannot be stably provided enough to perform the research and development thereof as medicines, and this fact has caused a big hindrance against the development on the research of such monocyte chemotactic factor.

Summary of the Invention

As the result of intensive investigations on the mass production of a monocyte chemotactic factor by direct expression system using E. coli as a host, the present inventors have found that the monocyte chemotactic factor can be massively prepared in an insoluble form within the transformed cells by using an improved expression plasmid for highly efficient production and a selected host strain of E. coli. Accordingly, the process of the present invention consists in using (1) a selected host strain of E. coli and (2) an expression plasmid to produce a monocyte chemotactic factor highly efficiently (3) in a high yield (4) as an unfused polypeptide (5) in an insoluble form within the cells, and it enables to provide massively said polypeptide at low costs as an economical and efficient mass production.

An object of the present invention is to provide a process for the production of a monocyte chemotactic factor polypeptide having monocyte chemotactic activity consisting of an amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence which has truncated 1-6 N-terminal amino acids by applying recombinant DNA technology using as a

host a selected E. coli or a mutant strain thereof which is transformed with an expression plasmid for highly efficient production.

Another object of the present invention is to provide a selected E. coli or a mutant strain thereof which is transformed with an expression plasmid for highly efficient production.

Detailed Description of the Invention

The present invention relates to an efficient process for producing a polypeptide having monocyte chemotactic activity and its transformant which comprises using an expression plasmid having a translation termination codon and a terminator sequence downstream of the structural gene and a translation initiation codon, a ribosome binding site sequence (hereinafter referred to as "SD sequence") and a promotor sequence derived from E. coli connected upstream of the structural gene; and using as a host E. coli, an E. coli LE392 strain, an E. coli BL21(DE3) strain, an E. coli AB1899 strain, an E. coli B/r-WP2-hcr$^-$ strain or an E. coli C600 hflA strain, or a mutant thereof; in the production of a polypeptide as an unfused polypeptide having monocyte chemotactic activity by using an expression plasmid in which there has been inserted DNA which encodes a polypeptide having monocyte chemotactic activity consisting of an amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence which has truncated 1-6 N-terminal amino acids thereof (this polypeptide will be hereinafter referred to as "monocyte chemotactic factor or monocyte chemotactic factor polypeptide"), transforming E. coli as a host by introducing said expression plasmid thereinto and cultivating the transformed E. coli.

The present invention will be below explained in detail.

An expression plasmid for producing the monocyte chemotactic factor can be constructed in accordance with fundamental theory on recombinant DNA technology and expression (for example, Maniatis, T. et al,

Molecular Cloning, a laboratory manual; Publn. Cold spring Harbor Laboratory, 1982). Thus, it can be constructed by connecting a SD sequence, a translation initiation codon and DNA encoding the monocyte chemotactic factor with a translation termination codon downstream of an appropriate promotor sequence (for example, trp, lac, PL, etc.), connecting a terminator sequence downstream thereof, optionally connecting an appropriate repressor gene derived from E. coli, and inserting it into appropriate vector (for example, pBR322, etc.) replicable in a host strain.

DNA encoding the monocyte chemotactic factor is known and can be prepared according to the method of Furutani, Y. et al, Biochem. Biophys. Res. Commun., 159, 249-255, 1989 or by chemical synthesis. Further, DNA encoding the monocyte chemotactic factor which has truncated 1-6 amino acids at the N-terminus can be constructed by deleting the corresponding DNA in according with the method for side-specific mutagenesis [for example, Kunkel. T.A. et al, Methods in Enzymol., 154, 367-382, 1987].

As for the terminator sequence in constructing the expression plasmid, the base sequence represented by SEQ ID NO:2 and a base sequence inclusive of said sequence are usable, and the former sequence is preferred.

Further, the sequence represented by SEQ ID NO:3 is preferred as the sequence from the SD sequence to the translation initiation codon (including the translation initiation codon).

A tryptophan promotor operator derived from E. coli is preferred as the promotor sequence. As for the repressor gene, a tryptophan promotor operator repressor gene (hereinafter referred to as "trpR gene")is preferred derived from E. coli such as a base sequence represented by SEQ ID NO:4.

Accordingly, as for an expression plasmid, there are preferable those having the translation termination codon and the above mentioned preferable terminator sequence downstream of DNA encoding the monocyte chemotactic factor, and the above mentioned preferable sequence from SD sequence to the translation initiation codon and the preferable promotor sequence connected upstream thereof. Specifically, preferred is an expression plasmid having the translation termination codon and the terminator sequence represented by SEQ ID NO:2 connected downstream of DNA encoding the monocyte chemotactic factor, and the sequence represented by SEQ ID NO:3 (sequence from the SD sequence to the translation initiation codon) and the tryptophan promotor operator derived from E. coli upstream thereof.

When an E. coli strain lacking in trpR gene such as the E. coli LE392 strain or a mutant strain thereof is used, an expression plasmid with trpR gene represented by SEQ ID NO:4 inserted is specially preferred.

The constructed expression plasmid is introduced into a selected strain of E. coli according to the calcium chloride method [Cohen, S.N. et al. Proc. Natl. Acad. Sci. USA, 69, 2110-2114, 1972], and the transformed E. coli is cultured in a conventional manner to produce and accumulate the monocyte chemotactic factor in the cultivated cells.

The selected strain of E. coli usable herein illustratively includes 6 strains of E. coli as shown below and mutant strains thereof.

(1) E. coli LE392 (ATCC 33572)

(2) E. coli P2392

[Raleigh, E.A. et al. Current Protocols in Molecular Biology, Edited by Ausubel, F.M., Greene Publishing Associates and Wiley-Interscience, New York, Unit 1.4, 1989].

(3) E. coli BL21(DE3)

[Studier, F.W et al, J. Mol. Biol., 189, 113-130, 1986]

(4) E. coli AB1899 (ME 8082)

(5) E. coli B/r-WP2-hcr$^-$ (ATCC 23233)

(6) E. coli C600 hflA

[Young, R.A. et al, Proc. Natl. Acad. Sci., USA, 80, 1194-1198, 1983]

Among the above mentioned strains of E. coli, the E. coli LE392 strain, the E. coli B/r-WP2-hcr$^-$ strain and the E. coli AB1899 strain are preferred, and the E. coli LE392 strain is most preferred.

Then, the cultivated cells are disrupted, and the pellet fraction recovered from the cell-lysate is treated with a mixture of low concentrated surfactant and a protein-denaturing agent, followed by treating with deoxyribonuclease. Thereafter, a pellet fraction containing the monocyte chemotactic factor was collected. The pellet fraction is solubilized with high concentrated protein-denaturing agent and subjected to dialysis or the like for removing the protein-denaturing agent to recover the monocyte chemotactic factor as a soluble polypeptide.

Specifically, the transformed E. coli is cultivated, and the cultivated cells containing the monocyte chemotactic factor are disrupted. The pellet fraction is recovered from the cell-lysate. This pellet fraction is washed with a mixture of low concentrated surfactant and a protein-denaturing agent (for example, 0.75 M urea containing 1% Triton X-100) and digested with deoxyribonuclease, whereby a pellet fraction containing the monocyte chemotactic factor is recovered. The pellet fraction is treated with high concentrated protein-denaturing agent (for example, 6 M guanidine hydrochloride) to solubilize the monocyte chemotactic factor. Then, the monocyte chemotactic factor can

be recovered in the form of a soluble polypeptide by removing the protein-denaturing agent, by dialysis or lowering the concentration of the protein-denaturing agent by dilution.

A highly purified preparation can be obtained by subjecting to chromatographies using heparin column and ion exchange column and gel filtration. Further, other methods such as ultrafiltration, electrophoresis, affinity chromatography using specific antibody and high-performance liquid chromatography using reverse-phase column can be effective for purification. The fundamental technique for purification can be performed according to the method reported by Furuta et al. [Furuta, R. et al., J. Biochem., 106, 436-441, 1989].

According to the process of the present invention, the monocyte chemotactic factor polypeptide is accumulated in a high content in the cells and recovered in the form of an insoluble polypeptide. Specifically, the productivity of the monocyte chemotactic factor polypeptide consisting of 76 amino acids represented by SEQ NO:1 [hereinafter referred to as "MCF(76)"] in using the E. coli LE392 strain as a host cell was about 3 mg per 100 ml of culture broth. Further, the productivity of MCF(76) with transformants of the E. coli P2392 strain and the E. coli BL21(DE3) strain was about 2-3 mg/100 ml (culture broth), that with transformants of the E. coli AB1899 strain and the E. coli B/r-WP2-hcr$^-$ strain was about 1 to 2 mg/100 ml (culture broth), and that with the transformant of the E. coli C600 hflA strain was about 0.5 to 1 mg/100 ml (culture broth).

The monocyte chemotactic factor accumulated in the cultivated cells was detected by SDS-polyacrylamide gel electrophoresis and the protein staining method with Coomassie Brilliant Blue-R-250 (hereinafter referred to as "CBB"). This assay is hereinafter referred to as "electrophoresis-CBB staining method". The accumulated amount was

determined densimetrically based on the protein band stained with CBB.

On the other hand, in the cases using as a host the E. coli strains other than those used in the process of the present invention, MCF(76) in the cultivated cells was rapidly degraded, therefore MCF(76) product was not detected in the cells by the electrophoresis-CBB staining method. Thus, as the results of cultivating the following 26 strains of E. coli as a host, no accumulation of MCF(76) was confirmed.

(1) W3110 (ATCC 27325)

(2) Q13 (ATCC 29079)

(3) x 1776 (ATCC 31244)

(4) RR1 (ATCC 31343)

(5) C600r^-m^- (ATCC 33525)

(6) HB101 (ATCC 33694)

(7) MC4100 (ATCC 35695)

(8) JM105 (ATCC 47016)

(9) JM109 (ATCC 53323)

(10) KL-16-19 (ME 6252)

(11) KL-16 (ME 8002)

(12) YN2092 (ME 8058)

(13) CSH29 (ME 8117)

(14) CSH22 (ME 8131)

(15) LS6745 (ME 8398)

(16) KD2157 (JE 8448)

(17) JRG902 (JE 6642)

(18) LC158 (ME 8476)

(19) N4830

(Gottesman, M.E. et al., J. Mol. Biol., 140, 57-75, 1980)

(20) MC4100htpR^-

(Yura, T. et al., Proc. Natl. Acad. Sci. USA, 81, 6803-6807, 1984)

(21) JC1552-03

(Yamagishi, J. et al., Mol. Gen. Genet., 204, 367-373, 1986)

(22) QD5003

(Yamagishi, J. et al. J. Bacteriol., 148, 450-458, 1981)

(23) CJ236

(Sambrook, J. et al., Molecular Cloning, a laboratory manual, 3, A.9, Cold Spring Harbor Laboratory Press (Publisher), 1989)

(24) DH5

(Sambrook, J. et al., Molecular Cloning, a laboratory manual, 3, A.10, Cold Spring Harbor Laboratory Press (Publisher), 1989)

(25) MV1184

(Sambrook, J. et al., Molecular Cloning, a laboratory manual, 3, A.11, Cold Spring Harbor Laboratory Press (Publisher), 1989)

(26) XL1-Blue

(Sambrook, J. et al., Molecular Cloning, a laboratory manual, 3, A.12, Cold Spring Harbor Laboratory Press (Publisher), 1989)

ATCC number described together with names of E. coli strains is Code No. of American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852, U.S.A.) and ME number or JE number are Code No. of National Institute of Genetics, Genetic Stocks Research Center (Tanida 1111, Mishima-shi, Shizuoka Prefecture, 411 Japan).

Mutant strains of E. coli used in the process of the present invention will be explained below.

The specific examples of mutant strains of E. coli are antibiotic resistant strains such as a streptomycin resistant mutant strain (hereinafter referred to as "Str^r") and a rifamycin resistant mutant strain (hereinafter referred to as "Rif^r"), a mutant strain conferring requirement for thymine (hereinafter referred to as "Thy^-"), a mutant strain conferring mutation of recA gene [Gudas, L.J. et al., Proc.

Natl. Acad. Sci. USA, 74, 5280-5284, 1977] (hereinafter referred to as "RecA$^-$"), and mutant strains conferring two or more of those properties at a time. Further, the mutant strains of E. coli usable in the process of the present invention include those conferring mutation to chromosome genes of these parent strains according to the method using N-methyl-N'-nitro-N-nitrosoguanidine [Miller, J.H., Experiments in Molecular Genetics, 125-129, Cold Spring Harbor Laboratory (Publisher), 1972]. Illustratively, mutant strains usable in the process of the present invention are obtained as follows: at first mutant strains are obtained by cultivating a parent strain in the presence of N-methyl-N'-nitro-N-nitrosoguanidine. Secondly, the above mutant strains are transformed with the expression plasmid for producing the monocyte chemotactic factor and the transformed strains are cultivated. Lastly, the mutant strains usable in the process of the present invention are obtained by selecting the mutant strains in which the monocyte chemotactic factor is accumulated by the cultivation. Furthermore, lysogenic strain for bacteriophage is also included in the mutant strains of E. coli usable in the process of the present invention. For example, mutant strains of the E. coli LE392 strain include the E. coli P2392 strain, a lisogenic strain for P2 phage of the E. coli LE392 strain. Moreover, mutant strains conferring new properties to those mutant strains include in mutant strains of E. coli usable in the process of the present invention, as far as the monocyte chemotactic factor can be detected by SDS-polyacrylamide gel electrophoresis and the protein staining method with CBB in the cultivated cells of the strain transformed with the expression plasmid.

These mutant strains can be prepared in a conventional manner of these technical field [Miller, J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory (Publisher), 1972].

The most preferable mutant strain used in the present invention is E. coli LE392 (Rif^r, RecA$^-$), namely, a mutant strain of the E. coli LE392 strain conferring rifamycin-resistant and recA gene mutation.

Preparation of Rif^r, Str^r, Thy$^-$ and RecA$^-$ of E. coli usable in the process of the present invention will be explained below.

An E. coli strain usable in the process of the present invention is incubated at 37 ℃ overnight on LB medium and centrifuged. The collected cells are suspended in saline, and the suspension are spread over the surface of LB agar plate containing rifamycin and incubated at 37℃ overnight. The colonies formed on LB agar plate are suspended in saline, streaked on LB agar plate containing rifamycin and incubated at 37℃ overnight, whereby an E. coli (Rif^r) strain is isolated as a single clone. Further an E. coli (Str^r) strain is also isolated by using LB agar plate containing streptomycin in place of LB agar plate containing rifamycin.

The culture of the E. coli (Rif^r) strain is inoculated at 5% concentration to M9 medium containing thymine, trimethoprim and L-methionine and cultivated at 37℃ overnight under shaking. This culture is inoculated at 5% concentration to a fresh medium having the same composition and incubated at 37℃ overnight under shaking. This culture is spread over LB agar plate containing thymine and incubated at 37℃ overnight. The colonies formed are collected, and their requirement for thymine are assayed. The colony showing requirement for thymine is suspended in saline, streaked on LB agar plate containing thymine and cultivated at 37℃ overnight, whereby an E. coli (Rif^r, Thy$^-$) strain is isolated as a single clone. Further, an E. coli (Str^r, Thy$^-$) strain is isolated as a single clone by using the E. coli (Str^r) strain in place of the E. coli (Rif^r) strain.

An E. coli KL-16-19 strain is incubated in LB medium at 37℃

overnight. This culture is inoculated at 10% concentration in LB medium and incubated at 37℃. On the other hand, the E. coli (Rif^r, Thy$^-$) strain is incubated in LB medium supplemented with thymine. The culture of the E. coli KL-16-19 strain is mixed with the culture of the E. coli (Rif^r, Thy$^-$) strain and incubated at 37℃ to cross a male strain, the E. coli Kl-16-19 strain with a female strain, the E. coli (Rif^r, Thy$^-$) strain. Then, the recombinant strain of which the requirement for thymine of the E. coli (Rif^r, Thy$^-$) strain reverted to the non-requirement for thymine is obtained by collecting the colony grown on M9 agar plate not containing thymine, and the obtained colony is streaked on LB agar plate and incubated at 37℃ overnight to isolate a single clone. Finally, an E. coli (Rif^r, RecA$^-$) strain is isolated by assaying sensitivity to U.V. (ultraviolet rays) of this isolated clone and selecting the clone conferring high sensitivity to U.V. because recA mutant gene derived from the E. coli KL-16-19 strain is inserted into the chromosome. In addition, a single clone, an E. coli (Str^r, RecA$^-$) strain is also isolated by treating the E. coli (Str^r, Thy$^-$) strain in place of the E. coli (Rif^r, Thy$^-$) strain according to the above method.

The productivity of the monocyte chemotactic factor polypeptide MCF(76) and a polypeptide which truncated N-terminal 1 amino acid, 3 amino acids or 6 amino acids, respectively, from MCF(76) using a mutant strain, for example, the E. coli (Rif^r, RecA$^-$) strain was equal or more than that in the case using the E. coli LE392 strain.

According to the process of the present invention, the monocyte chemotactic factor, namely MCF(76), and a polypeptide which truncated N-terminal 1 amino acid, 3 amino acids or 6 amino acids, respectively, from MCF(76) can be efficiently produced.

The following abbreviations are used for simplifying the

description of the present specification.

ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
SD sequence	Shine-Dalgano sequence (ribosome binding site sequence)
SDS	sodium laurylsulfate
IL-1α	Interleukin-1α
Str^r	resistance to streptomycin
Rif^r	resistance to rifamycin
Thy$^-$	requirement for thymine
RecA$^-$	mutation of recA gene
DMSO	dimethyl sulfoxide
trpR gene	tryptophan promotor operator repressor gene

The following Examples illustrate embodiments of the present invention, but it should be noted that present invention is in no way limited to these examples.

The fact that the polypeptides produced in the following Examples are the monocyte chemotactic factor has been identified from the fact that these show strong monocyte chemotactic activity, measurement of the molecular weight by SDS-polyacrylamide gel electrophoresis analysis and the determination of the amino acid sequence at the N-terminal region according to the Edman's degradation method using Protein Sequencer (470A type; Applied Biosystem, U.S.A.). Monocyte chemotactic activity was assayed according to the method of Matsushima, K. et al. [J. Exp. Med., 169, 1485-1490, 1989].

Example 1

Construction (1) of an expression plasmid for producing the monocyte

chemotactic factor

An expression plasmid pHM483 for producing the monocyte chemotactic factor MCF(76) consisting of 76 amino acids represented by SEQ ID NO:1 was constructed by the following method.

An expression plasmid pHMC076 (cf. Referential Example 1 shown below) was digested with restriction enzymes SpeI and SalI to isolate the DNA fragment of about 0.6 kilo base pair (kbp) having the base sequence represented by SEQ ID NO:5. This DNA fragment is referred to as "MCF(SS)" fragment. Recombinant phage DNA was prepared by inserting this MCF(SS) fragment between the cleavage sites of restriction enzymes SalI and XbaI in the multi-cloning sites of M13mp19 phage vector (Takara Shuzo Co.). One base (G) was inserted at the site between base No. 278 and 279 (or base No. 279 and 280) of the base sequence of MCF(SS) fragment represented by SEQ ID NO:5, using this recombinant phage DNA and mutagenic primer of the base sequence represented by SEQ ID NO:6 according to the following method based on the site-specific mutagenic method [Kunkel, T.A. et al., Methods in Enzymol., 154, 367-382, 1987]. This site-specific mutagenesis was performed with a Muta-Gene in vitro mutagenesis kit (Bio Rad Labs., U.S.A.). Thus, said recombinant phage DNA was transfected to E. coli JM105, which was incubated to give the phage. Then, this phage was transfected to E. coli CJ236, which was incubated on 2xTY medium [1.6% tryptone, 1% yeast extract, 0.5% sodium chloride] containing uridine (1μg/ml) and chloramphenical (20μg/ml) at 37℃ for 5 hours, and single-stranded phage DNA into which uracil was introduced was isolated from the supernatant.

Phosphorylated mutagenic primer in which phosphate group was added to its 5'-terminus with T4 polynucleotide kinase was allowed to hybridize with the single-stranded phage DNA containing uracil prepared above in annealing buffer [20 mM Tris-HCl buffer (pH 7.4) containing 2

mM magnesium chloride and 50 mM sodium chloride] at 70 ℃ for 10 minutes and the mixture was gradually chilled up to 30℃ at a rate of 1℃/minute to hybridize the primer to phage DNA. Then, phage DNA was allowed to react with T4 DNA polymerase in synthesis buffer [10 mM Tris-HCl buffer (pH 7.4) containing each 0.4 mM of four deoxyribonucleotide triphosphates (dGTP, dCTP, dATP and dTTP), 0.75 mM ATP, 3.75 mM magnesium chloride and 1.5 mM dithiothreitol] under reaction conditions on ice for 5 minutes, at 25 ℃ for 5 minutes and at 37℃ for 90 minutes to synthesize complementary DNA to the template phage DNA, and the termini of the complementary DNA were ligated by T4 DNA ligase, and the reaction was stopped by freezing at -20℃ in order to prepare Double-stranded closed-circular DNA. The obtained DNA was transfected to E. coli JM105 and incubated to isolate a double-stranded replicative form of DNA in which the mutation was introduced (hereinafter referred to as "mutated double-stranded DNA"). The mutated double stranded DNA was digested with restriction enzymes DraI and SalI to isolate DNA fragment having the base sequence represented by SEQ ID NO:7. This DNA fragment is referred to as "MCF(DS)". In the base sequence of this MCF(DS) fragment, the recognition sequence (GGATCC) at base No. 248-253 of SEQ ID NO:7 with restriction enzyme BamHI was constructed by inserting one base (G) due to the method above.

Separately, the expression plasmid pHMC076 was digested with restriction enzyme DraI and SalI to cut into two fragments, whereby there was isolated a larger DNA fragment (hereinafter referred to as "HMC076 vector fragment") including a tryptophan promotor sequence derived from E. coli (hereinafter referred to as "trp promotor"), an ampicillin-resistant gene, and a replication origin but not including the structural gene of the monocyte chemotactic factor MCF(76). The HMC076 vector fragment was ligated with T4 DNA ligase to MCF (DS)

fragment to construct an expression plasmid pHMC076-B.

The expression plasmid pHMC076-B was digested with restriction enzymes XhoI and BamHI to remove 5 bases (TCGAG, fragment corresponding to base No. 244-248 of the base sequence represented by SEQ ID NO:7) between the cleavage sites of XhoI and BamHI, and a double-stranded DNA prepared by annealing two chemically synthesized DNAs represented by SEQ ID NO:8 and 9 was integrated in this site to construct an expression vector pHM483. 42 bases except for 4 bases at 5'-terminal side of the chemically synthesized DNAs represented by the base sequence of SEQ ID NO:9 was a complementary strand of chemically synthesized DNA represented by the base sequence of SEQ ID NO:8, and both DNA fragments were annealed to obtain the following double-stranded DNA represented by SEQ ID NO:10 having the cohesive end of restriction enzyme XhoI and that of restriction enzyme BamHI at both ends, respectively.

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5'-TCGAGTAGCCCGCCTAATGAGCGGGCTTTTTTTTCGCTTGAATTCG
    3'-CATCGGGCGGATTACTCGCCCGAAAAAAAAGCGAACTTAAGCCTAG
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The sequence of this DNA includes the base sequence represented by SEQ ID NO:2 and was set so as to function as a terminator, namely signal for terminating transcription. The base sequence was determined according to the dideoxy method using 7-DEAZA sequencing kit (Takara Shuzo Co.).

Example 2

Construction (2) of expression plasmids for producing the monocyte chemotactic factor

Expression plasmids for producing the monocyte chemotactic factor which truncated N-terminal 1-6 amino acids from MCF(76) were constructed as follows. Polypeptides which truncated N-terminal 1 amino acid, 3 amino acids and 6 amino acids from MCF(76) are referred to as

"MCF(75), MCF(73) and MCF(70)", respectively. Expression plasmids for producing these 3 polypeptides which truncated N-terminal amino acids are referred to as "pHM484, pHM485 and pHM486", respectively in that order.

(1) Construction of an expression plasmid pHM484

An expression plasmid pH484 for producing the monocyte chemotactic factor MCF(75) was constructed by the following method. The expression plasmid pHM483 obtained by the method obtained in Example 1 was digested with restriction enzymes SpeI and SalI to isolate DNA fragment containing the region encoding MCF(76). This DNA fragment was inserted between the cleavage sites of restriction enzyme SalI and XbaI in the multi-cloning sites of M13mp19 phage vector to construct recombinant phage DNA(MCF76). Using this recombinant phage DNA(MCF76), the codon (CAG) corresponding to the N-terminal amino acid (Gln) of MCF(76) was deleted according to the site-specific mutagenic method described in Example 1. As a mutagenic primer was used chemically synthesized DNA having the base sequence represented by SEQ ID NO:11. The resultant mutated double-stranded DNA was digested with restriction enzymes DraI and XhoI to isolate the mutated DNA fragment which truncated the codon corresponding to the N-terminal amino acid (Gln) of MCF(76).

Separately, the expression plasmid pHM483 constructed by the method as described in Example 1 was digested with restriction enzymes DraI and XhoI to cut into two fragments to isolate a larger DNA fragment (hereinafter referred to as "HM483 vector fragment") including the trp promotor, the terminator sequence, the ampicillin-resistant gene, and the replication origin but not including the structural gene of the monocyte chemotactic factor MCF(76). This HM483 vector fragment was ligated to said mutated DNA fragment with T4 DNA ligase to construct the expression plasmid pHM484. The base sequence of the expression plasmid

pHM484 corresponded to the sequence which truncated the codon corresponding to the N-terminal amino acid (Gln) of MCF(76) from the base sequence of the expression plasmid pHM483. The base sequence was determined by the dideoxy method using 7-DEAZA sequence kit.

(2) Construction of other expression plasmids

Expression plasmids pHM485 and pHM486 for producing the polypeptide, namely MCF(73) and MCF(70) which truncated N-terminal 3 amino acids and 6 amino acids from MCF(76) were constructed with recombinant phage DNA(MCF76) and the respectively corresponding mutagenic primers according to the method described in above section (1). As mutagenic primers were used the respective chemically synthesized DNA corresponding to the base sequence encoding the peptide to be truncated.

An expression plasmid pHM485 was constructed as follows: the mutated double-stranded DNA which truncated the base sequence (CAGCCAGAT) encoding the N-terminal 3 amino acids (GlnProAsp) of MCF(76) was obtained by using recombinant phage DNA(MCF76) and a mutagenic primer represented by SEQ ID NO:12 according to the site-specific mutagenic method. Then, the mutated DNA fragment obtained by digesting the mutated double-stranded DNA with restriction enzyme DraI and XhoI was ligated to said HM483 vector fragment.

An expression plasmid pHM486 was constructed as follows: the mutated double-stranded DNA which truncated the base sequence (CAGCCAGATGCAATCAAT) encoding N-terminal 6 amino acids (GlnProAspAlalleAsn) of MCF(76) was obtained by using recombinant phage DNA(MCF76) and a mutagenic primer represented by SEQ ID NO:13 according to the site-specific mutangenesis. Then, the mutated DNA fragment obtained by digesting the mutated double-stranded DNA with restriction enzymes DraI and XhoI was ligated to HM483 vector fragment.

Example 3

Construction (3) of an expression plasmid for producing monocyte chemotactic factor

An expression plasmid pHM583 for producing the monocyte chemotactic factor MCF(76) in which trpR gene had been inserted was constructed by the following method.

Chromosomal DNA was extracted from E. coli HB101 according to the following method as modified from Cosloy et al., Mol. Gen. Genet., 124, 1-10, 1973. Thus, the cultivated cells were suspended in 0.1 M sodium chloride solution containing 0.1 M disodium ethylenediamine-tetraacetate solution, mixed with 50 mM Tris-HCl buffer (pH 8.0) containing pronase K (50μg/ml) and 0.5% SDS and heated at 42 ℃ for 1 hour to dissolve the cells and digest the bacterial protein. Then, phenol was added to the reaction mixture to remove protein, and ribonuclease A (50μg/ml) was added to the recovered aqueous phase and heated at 37℃ for 30 minutes to degrade RNA. Finally, ethanol was added to the reaction mixture to recover the chromosomal DNA of E. coli.

The resulting chromosomal DNA was digested with restriction enzyme NlaIV to isolate about 1kbp fragment according to the low-melting-temperature agarose gel electrophoresis method. This DNA fragment was inserted between the cleavage site of restriction enzyme SmaI in the multi-cloning site of M13mp18 phage vector to prepare recombinant phage DNA library.

DNA fragment including trpR gene represented by SEQ ID NO:15 was cloned with a probe having the base sequence (corresponding to the sequence from base No. 363 to 376 of SEQ ID NO:15) represented by SEQ ID NO:14 from this library according to the following method as modified from the plaque-hybridization method [Sambrook, J. et al., Molecular

Cloning, a Laboratory Manual, Vol. 1, 2.108, Cold Spring Harbor Laboratory Press (Publisher), 1989].

According to the plaque-hybridization method, said recombinant phage DNA library was at first infected to E. coli JM105 to form plaques. Then, about 500 single plaques were individually infected to E. coli JM105, and the strains infected were incubated to recover the resulting phage. Thus prepared phages were dot-blotted to nitrocellulose filters, which were immersed in 1.5 M sodium chloride solution containing 0.1N sodium hydroxide, allowed to stand still at room temperature for 5 minutes and neutralized by soaking in 0.5 M Tris-HCl buffer (pH 7.0) containing 3 M sodium chloride at room temperature for 10 minutes. This filter was baked at 80℃ for 2 hours to fix phage DNA thereon.

Labelling with [γ -^{32}P]ATP of the probe represented by SEQ ID NO:14 was prepared by the 5'-end-DNA labelling method using MEGALABEL kit (Takara Shuzo Co.).

The filter on which said recombinant phage DNA was fixed was allowed to incubated at about 40℃ for 48 hours in 5 fold-concentrated SSC (a solution of 0.15 M sodium chloride containing 0.015 M sodium citrate is referred to as "SSC") containing ^{32}P-labeled probe (about 1,000,000 cpm/ml), denatured salmon sperm DNA (100μg/ml), 0.1% SDS, 1 mM disodium ethylenediamine tetraacetate, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone and 0.02% ficoll.

Then the filtrate was heated at 65℃ for 1 hour in 5-fold-concentrated SSC containing 0.1% SDS and washed 3 times with 2 fold-concentrated SSC. The filter was dried and subjected to autoradiography to detect the phage which was bound to ^{32}P-labeled probe. A recombinant phage corresponding to this phage DNA is referred to as "candidate phage". The candidate phage was obtained at a frequency of about one

per 100 phages from the recombinant phage DNA library. Then, single-stranded phage DNA was isolated from the candidate phage by the conventional method, and the base sequence of this single-stranded phage DNA was determined by the dideoxy method using 7-DEAZA Sequence Kit, and it was confirmed that the selected recombinant phage inclusive of trpR gene represented by SEQ ID NO:15. This recombinant phage is hereinafter referred to as "M13mp18-trpR".

Using M13mp18-trpR, the cleavage recognition sequence (GAATTC) of restriction enzyme EcoRI was inserted between base No. 116 and base No. 117 of SEQ ID NO:15, and the cleavage recognition sequence (GGATCC) of restriction enzyme BamHI was inserted between base No. 956 and base No. 957 of SEQ ID NO:15 according to the site-specific mutagenesis method as described in Example 1.

Chemically synthesized DNAs having the base sequences of SEQ ID NO: 16 and SEQ ID NO:17 were used as a mutagenic primer.

The resultant mutated double-chain DNA was digested with restriction enzymes EcoRI and BamHI to isolate trpR gene fragment (SEQ ID NO:4) having the sequence of the cohesive end of EcoRI at the 5'-end of the sequence of base No. 117 to 956 of SEQ ID NO:15 and having the sequence of the cohesive end of BamHI at the 3'-end. This trpR gene fragment is referred to as "TRP(EB) fragment".

Separately, the expression plasmid pHM483 constructed by the method as described in Example 1 was digested with restriction enzymes EcoRI and BamHI to cut into two fragments, whereby a larger DNA fragment including the trp promotor, the terminator sequence, the ampicillin resistant gene, the structural gene of the monocyte chemotactic factor, and the replication origin was isolated. This DNA fragment is referred to as HM483(EB) vector fragment. Said TRP(EB) fragment was ligated this vector fragment with T4 DNA ligase to construct the expression

plasmid pHM583. The base sequence of the expression plasmid pHM583 was corresponded to the sequence which deleted 6 bases (AATTCG, the fragment corresponding to base No. 41 to 46 of the base sequence of SEQ ID NO: 8) between the cleavage sites of EcoRI and BamHI of the expression plasmid pHM483 and which inserted TRP(EB) fragment represented by SEQ ID NO:4 into the above deleted site. Its base sequence was determined by the dideoxy method using 7-DEAZA Sequence Kit.

Example 4

Construction (4) of expression plasmids for producing the monocyte chemotactic factors

Expression plasmids for producing the monocyte chemotactic factors MCF(75), MCF(73) and MCF(70) into which trpR gene was inserted were constructed by the following method. These 3 expression plasmids are referred to as "pHM584, pHM585 and pHM586", respectively in that order.

Expression plasmids pHM484, pHM485 and pHM486 constructed by the method as described in Example 2 were digested with restriction enzymes EcoRI and BamHI to isolate a larger DNA fragments containing the region encoding MCF(75), MCF(73) and MCF(70), respectively.

These three vector fragments were ligated TRP(EB) fragment as prepared by the method of Example 3 with T4 DNA ligase to construct expression plasmids pHM584, pHM585 and pHM586, respectively. The base sequence of these expression plasmids were determined by the dideoxy method using 7-DEAZA Sequence Kit.

Example 5

Production (1) of the monocyte chemotactic factor MCF(76)

The monocyte chemotactic factor MCF(76) was prepared by introducing the expression plasmid PHM483 into the E. coli LE392 strain according to the following method.

The E. coli LE392 strain was inoculated to LB medium [1% tryptone,

0.5% yeast extract, 1% sodium chloride (pH 7.2)] and incubated at 30 ℃ until the OD 600 nm reached about 0.5. This culture was allowed to stand still for 30 minutes on ice and centrifuged to obtain the cells. The cells were suspended in 50 mM calcium chloride solution, allowed to stand still on ice for 60 minutes and centrifuged to obtain the cells. The cells were suspended in 50 mM calcium chloride solution containing 20% glycerin. To this cells suspension was added the expression plasmid pHM483 constructed by the method of Example 1, and the resultant mixture was allowed to react on ice for 20 minutes and at room temperature for 10 minutes, mixed with LB medium and incubated at 37℃ for 60 minutes under shaking. This culture was spread over LB agar plate (agar concentration 1.5%) containing ampicillin (25μg/ml), incubated at 37℃ overnight, whereby there was selected a transformed E. coli conferring ampicillin resistance by introducing said plasmid. The resultant transformed E. coli is referred to as "E. coli LE392/pHM483".

E. coli LE 392/pHM483 was incubated at 37℃ overnight on LB medium, and the culture was inoculated at 1% concentration to a productive medium [composition: 1.5% disodium phosphate 12 hydrate, 0.3% monopotassium phosphate, 0.1% ammonium chloride, 2μg/ml vitamin B_1, 0.5% Casamino acid, 2 mM magnesium sulfate, 0.1 mM calcium chloride, 1% tryptone, 0.5% yeast extract, 1% sodium chloride, and 0.4% glycerin], added with 3-indoleacrylic acid to a final concentration of 20μg/ml, and incubated at 37℃ for about 24 hours. The cells collected by centrifugation were suspended in 20 mM Tris-HCl buffer (pH 7.5) and then disrupted by sonification to obtain a cell-lysate. The content of MCF(76) in this cell-lysate was assayed by the electrophoresis-CBB staining method.

As the result, a large amount of MCF(76) was detected in the cell-

lysate, and most of MCF(76) was recovered as an insoluble polypeptide in the pellet fraction after centrifugation of the cell-lysate. The content of MCF(76) in the total insoluble protein of the culture of E. coli LE392/pHM483 was not less than about 20%, and the productivity of MCF(76) was equivalent to about 3 mg per 100 ml culture. At the N-terminus of MCF(76) was detected a methionine residue due to the translation initiation codon and the methionine residue was found to have hardly been removed.

Example 6

Production (2) of the monocyte chemotactic factor MCF(76)

The monocyte chemotactic factor MCF(76) was prepared by incubating each transformant, made by introduction of the expression plasmid pHM483 constructed by the method of Example 1 according to the method of Example 5, using the E. coli P2392 strain, the E. coli BL21(DE3) strain, the E. coli AB1899 strain, the E. coli B/r-WP2-hcr$^-$ strain and the E. coli C600 hflA strain as a host cell. A large amount of MCF(76) was observed by the electrophoresis-CBB staining method in the cell-lysate of the transformants of the E. coli P2392 strain, the E. coli BL21(DE3) strain, the E. coli AB1899 strain, the E. coli B/r-WP2-hcr$^-$ strain and the E. coli C600 hflA strain. Its content was equivalent to about 2-3 mg/100 ml (culture broth) in the transformants of the E. coli P2392 strain and the E. coli BL21(DE3) strain, about 1-2 mg/100 ml (culture broth) in the transformants of the E. coli AB1899 strain and the E. coli B/r-WP2-hcr$^-$ strain, and about 0.5-1 mg/100 ml (culture broth) in the E. coli C600 hflA strain.

Example 7

Production (1) of the monocyte chemotactic factor in the E. coli LE392 (Rif^r, RecA$^-$) strain as a host cell

The monocyte chemotactic factor MCF(76) was prepared in the direct

expression system, using the E. coli LE392 (Rif^r, RecA$^-$) strain as a host cell made by the method of Referential Example 2 described below. Thus, the expression plasmid pHM483 constructed by the method of Example 1 was introduced into the E. coli LE392 (Rif^r, RecA$^-$) strain according to the method of Example 5, and the resultant transformed E. coli was incubated. It was confirmed that MCF(76) could be equally produced in the direct expression system, using the E. coli LE392 (Rif^r, RecA$^-$) strain as a host cell, compared with the case using E. coli LE392 strain by assaying the content of MCF(76) in the cell-lysate by the electrophoresis-CBB staining method. As in the case of the E. coli LE392 strain as a host cell, the methionine residue due to the translation initiation codon at the N-terminus of MCF(76) was detected by analyzing the N-terminal amino acid sequence and it was found that the methionine residue was hardly removed.

Example 8

Production (2) of the monocyte chemotactic factor in the E. coli LE392 (Rif^r, RecA$^-$) strain as a host cell

The monocyte chemotactic factors MCF(75), MCF(73) and MCF(70), namely N-terminal truncated polypeptide of MCF(76) were prepared in the direct expression system, using the E. coli LE392 (Rif^r, RecA$^-$) strain made by the method of Referential Example 2 described below as a host cell. Thus, expression plasmids (pHM484, pHM485 or pHM486) for producing each truncated polypeptide constructed by the method of Example 2 was introduced into the E. coli LE392 (Rif^r, RecA$^-$) strain according to the method of Example 5, and the resultant transformed E. coli were incubated. It was confirmed by assaying the productivity of each polypeptide in the cultivated cells according to the electrophoresis-CBB staining method that each of these polypeptides could be equally produced in the transformant, E. coli LE392 (Rif^r,

RecA⁻), compared with the case using the E. coli LE392 strain. Further, there was observed an inclination that the ratio of removing the methionine residue due to the translation initiation codon from the N-terminus of these monocyte chemotactic factor polypeptides would depend upon the N-terminal structure of each polypeptide. The methionine residue due to the translation initiation codon of the monocyte chemotactic factors MCF(75), MCF(73) and MCF(70) was found to have been almost completely removed.

Example 9

Production (3) of the monocyte chemotactic factors using the E. coli LE392 (Rifʳ, RecA⁻) strain as a host cell

The monocyte chemotactic factor MCF(76) and N-terminal truncated polypeptides, namely the monocyte chemotactic factors MCF(75), MCF(73) and MCF(70) were prepared in the direct expression system using the E. coli LE392 (Rifʳ, RecA⁻) strain as a host cell prepared in Referential Example 2 as described below.

Thus, expression plasmids (pHM583, pHM584, pHM585 and pHM586) for producing the various monocyte chemotactic factors in which trpR gene inserted by the method described in Example 3 and Example 4 were introduced into the E. coli LE392 (Rifʳ, RecA⁻) strain according to the method of Example 5, and each transformed E. coli was cultivated. It was confirmed that said polypeptide was equally or more produced in the transformed E. coli LE392 (Rifʳ, RecA⁻), compared with the case using the E. coli LE392 strain, by assaying the productivity of each monocyte chemotactic factor in each cultivated cell according to the electrophoresis-CBB staining method.

Its yield was equal to or more than those in the cases using pHM483, pHM484, pHM485 and pHM486 as an expression plasmid for producing each monocyte chemotactic factor. Further, the methionine residue due to the

translation initiation codon was found to have almost remained in the N-terminus of the monocyte chemotactic factor MCF(76), but the methionine residue due to the translation initiation codon was found to have almost completely removed in terms of MCF(75), MCF(73) and MCF(70).

Referential Example 1

Construction of an expression plasmid pHMC076

An expression plasmid pHMC076 for producing the monocyte chemotactic factor MCF(76) was constructed by the following method. DNA encoding MCF(76) was prepared by using a recombinant plasmid pHMCF7 [Furutani, Y. et al, Biochem. Biographys. Res. Commun., 159, 249-255, 1989]. The base sequence of cDNA inserted in a recombinant plasmid pHMCF7 was shown by SEQ ID NO:18. The recombinant plasmid pHMCF7 was digested with restriction enzyme PstI to isolate DNA fragment including the base sequence encoding MCF(76). This DNA fragment was inserted into the cleavage site of restriction enzyme PstI in the multi-cloning site of M13mp18 phage vector (Takara Shuzo Co.). The base sequence represented by SEQ ID NO:21 was inserted upstream of the codon (CAG) corresponding to the N-terminal amino acid (Gln) of MCF(76), using this recombinant phage DNA and two chemically synthesized DNAs having the base sequence represented by SEQ ID NO:19 and 20 as each mutagenic primer, according to the site-specific mutagenesis method described by Example 1, and further the base sequence of 5'-TGACTCGAG-3' was inserted downstream of the termination codon continuing to the C-terminal amino acid (Thr). The resultant mutated double-stranded DNA is referred to as "(DraI)(XhoI) inserted phage DNA". The partial base sequence is shown in SEQ ID NO:22. This mutated double-stranded DNA was digested with restriction enzymes DraI and XhoI to isolate DNA fragment corresponding to the 142-384th of the base sequence represented by SEQ ID NO:22. This DNA fragment is referred to as "MCF

(DraI-XhoI) fragment". This MCF (DraI-XhoI) fragment included the sequence having the translation initiation codon upstream of DNA encoding MCF(76) and the translation termination codon downstream thereof.

Separately, an expression vector pEP205 [Furuta, R. et al., J. Biochem., 106, 436-441, 1989] was digested with restriction enzymes DraI and XhoI to isolate a large DNA fragment completely free of human IL-1α structural gene inserted in pEP205. This DNA fragment is referred to as EP205 vector fragment. The base sequence of EP205 vector fragment includes the trp promotor, the ampicillin-resistant gene and the replication origin. Said MCF (DraI-XhoI) fragment was ligated to EP205 vector fragment with T4 DNA ligase to construct the expression plasmid pHMC076. The base sequence was determined by the dideoxy method using 7-DEAZA sequence kit.

Further, the structure of the expression vector pEP205 was disclosed in Fig. 2 (p. 437) of Furuta, R. et al. report [J. Biochem., 106, 436-441, 1989]. This EP205 vector fragment corresponds to about 3.7 kbp of (d)-(a)-(b) region except for (c) region in the structure of Fig. 2. The (a)-(b) region includes the trp promotor and the SD sequence and is referred to as "E. coli trp-SD sequence". The base sequence is represented by SEQ ID NO:23. The (d) region is due to pBRS6 [Yamada, M. et al., J. Biotechnol., 3, 141-153, 1985], a derivative of pBR322, and free of the recognition sequence (3 sites) of restriction enzyme DraI. The complete base sequence of plasmid pBR322 has been determined [Sutcliffe, J. G., Cold Spring Harbor Symp. Quant. Biol., 43, 77-90, 1979; Peden, K. W. C., Gene, 22, 277-280, 1983].

Referential Example 2

Preparation of an E. coli LE392 mutant strain

Using the E. coli LE392 strain as a parent strain, a mutant strain

[hereinafter referred to as " E. coli LE392 (Rif^r)"] having rifamycin resistance (Rif^r), a mutant strain [hereinafter referred to as "E. coli LE392 (Rif^r, Thy$^-$)"] conferring requirement for thymine and a recA mutant strain [hereinafter referred to as "E. coli LE392 (Rif^r, RecA$^-$)"] having rifamycin resistance and U.V. high sensitivity by integrating recA mutant gene.

(1) Preparation of an E. coli LE392 (Rif^r) strain

The E. coli LE392 strain was incubated at 37℃ overnight under shaking in 10 ml of LB medium, and the cells collected by centrifugation were suspended in 1 ml of saline. The cell suspension (0.1 ml) was spread over LB agar plate containing rifamycin (100 μ g/ml) and incubated at 37℃ overnight. The colony formed on LB agar plate was suspended in saline, streaked on LB agar plate containing rifamycin (100 μ g/ml) and incubated at 37℃ overnight to isolate single colonies.

These colonies isolated were confirmed to have both properties, namely requirement for methionine due to the phenotype of the parent strain and rifamycin resistance specific to the mutant strain, and this strain was identified as " E. coli LE392 (Rif^r)". The rifamycin resistance was determined using as an index of growing on LB agar plate containing rifamycin (100 μ g/ml). The requirement for methionine was determined using an index of growing on M9 agar medium [composition: 1.5% disodium phosphate 12 hydrate, 0.3% monopotassium phosphate, 0.1% ammonium chloride, 2 μ g/ml vitamin B_1, 2 mM magnesium sulfate, 0.1 mM calcium chloride, 0.05% sodium chloride, 0.2% glucose and 1.5% agar] containing L-methionine (40 μ g/ml) but not growing on M9 agar medium free of L-methionine. The resultant E. coli LE392 (Rif^r) strain was incubated on LB medium containing rifamycin (100 μ g/ml), and this culture was mixed with dimethyl sulfoxide (DMSO) at 7% concentration and stored by

freezing.

(2) Preparation of an E. coli LE392 (Rif^r, Thy$^-$) strain

The culture of the E. coli LE392 (Rif^r) strain obtained by incubating overnight under shaking on LB medium containing rifamycin (100 μg/ml) was inoculated at 5% concentration to M9 medium [composition: 1.5% disodium phosphate 12 hydrate, 0.3% monopotassium phosphate, 0.1% ammonium chloride, 2 μg/ml vitamin B_1, 2 mM magnesium sulfate, 0.1 mM calcium chloride, 0.05% sodium chloride and 0.2% glucose] containing thymine (200 μg/ml), trimethoprim (200 μg/ml) and L-methionine (40 μg/ml) and incubated at 37℃ overnight under shaking. Then furthermore the culture was inoculated at 5% concentration to fresh M9 medium having the same composition and incubated at 37℃ overnight under shaking. The resultant culture was spread over LB agar plate containing thymine (200 μg/ml) and incubated at 37℃. Colonies formed were assayed on requirement for thymine. The requirement for thymine was determined with an index that allowed to grow on the M9 agar plate containing thymine (200 μg/ml) and L-methionine (40 μg/ml) but didn't allow to grow on the M9 agar plate containing L-methionine (40 μg/ml). The colony showing the requirement for thymine by said assay was suspended in saline, streaked on LB agar plate containing thymine (200 μg/ml) and incubated at 37℃ overnight to isolate single colonies. It was again confirmed that this clone isolated showed properties such as requirement for thymine, rifamycin resistance and requirement for methionine, whereby this strain was identified as an E. coli LE392 (Rif^r, Thy$^-$) strain. The E. coli LE392 (Rif^r, Thy$^-$) strain prepared was incubated on LB medium containing rifamycin (100 μg/ml) and thymine (200 μg/ml), and the resultant culture was mixed with dimethyl sulfoxide at 7% concentration and stored by freezing.

(3) Preparation of an E. coli LE392 (Rif^r, RecA$^-$) strain

The E. coli KL-16-19 strain was incubated still on LB medium at 37℃ overnight. This culture was inoculated at 10% concentration on LB medium and incubated at 37℃ under shaking very slowly. Incubation was stopped at the time when the turbidity of the culture at 600 nm wave length reached about 0.5 to give a culture equivalent to about 5×10^8 /ml of cell number. The E. coli KL-16-19 strain is available from National Institute of Genetics, Genetic Stocks Research Center, Mishima, 411 Japan and Stock No. of that institute is ME 6252.

The E. coli LE392 (Rif^r, Thy^-) strain was incubated on LB medium containing thymine (200μg/ml) under the same conditions as described above to give a culture having the same level of cell number. The resultant culture of the E. coli KL-16-19 strain was mixed with the culture of the E. coli LE392 (Rif^r, Thy^-) strain at a ratio of 1:10 and incubated at 37℃ under shaking very slowly to cross a male strain, the E. coli KL-16-19 strain with a female strain, the E. coli LE392 (Rif^r, Thy^-) strain. After incubation for 60 minutes, this mixed culture was chilled on ice, spread over M9 agar plate containing rifamycin (100μg/ml) and L-methionine (40μg/ml) and incubated at 37℃ for 2 days. The colony which grew over M9 agar plate free of thymine was selected to obtain a recombinant strain of the E. coli LE392 (Rif^r, Thy^-) strain of which requirement for thymine was reverted to non-requirement. This strain was suspended in saline, streaked over LB agar plate and incubated at 37℃ overnight to isolate single clones. It was again confirmed that one of the single clones isolated showed requirement for methionine and rifamycin resistance but free of requirement for thymine. Assays for these properties were as shown above.

Further, sensitivity to U.V. of the clone isolated was assayed, whereby the clone showing high sensitivity to U.V. by integrating recA mutant gene derived from the E. coli KL-16-19 strain was selected.

Assay for sensitivity to U.V. was performed by inoculating a strain on LB agar plate, irradiating U.V. (0.5 mW/cm^2) for 5 seconds, incubating at 37 ℃ overnight and deciding whether it grew or not. Any strain showing high sensitivity to U.V. was selected, using criteria that it didn't grow under irradiation of U.V. as described above under which conditions the E. coli LE392 strain, the E. coli LE392 (Rif^r) strain or the E. coli LE392 (Rif^r, Thy$^-$) strain were able to grow. The resultant strains showing high U.V. sensitivity are referred to as "E. coli LE392 (Rif^r, RecA$^-$) strain". E. coli LE392 (Rif^r, RecA$^-$) strain prepared was incubated on LB medium containing rifamycin (100μg/ml), mixed with dimethyl sulfoxide at 7% concentration and stored by freezing.

Referential Example 3

Preparation (1) of an E. coli B/r-WP2-hcr$^-$ mutant strain

Using the E. coli B/r-WP2-hcr$^-$ as a parent strain, an E. coli B/r-WP2-hcr$^-$ (Rif^r) strain showing rifamycin resistance and an E. coli B/r-WP2-hcr$^-$ (Rif^r, Thy$^-$) strain showing rifamycin resistance and requirement for thymine were prepared according to the method described in Referential Example 2.

Referential Example 4

Preparation (2) of an E. coli B/r-WP2-hcr$^-$ mutant strain

Using the E. coli B/r-WP2-hcr$^-$ strain as a parent strain, an E. coli B/r-WP2-hcr$^-$ (Str^r) strain showing streptomycin resistance and an E. coli B/r-WP2-hcr$^-$ (Str^r, Thy$^-$) strain showing streptomycin resistance and requirement for thymine were prepared according to the method described in Referential Example 2. In this case, streptomycin (100μg/ml) was used in place of rifamycin in each step for preparing mutant strains.

SAMPLE SEQUENCE LISTING

SEQ ID NO:1

SEQUENCE LENGTH:76

SEQUENCE TYPE:amino acid

TOPOLOGY:linear

MOLECULE TYPE:protein

SEQUENCE DESCRIPTION

```
Gln Pro Asp Ala Ile Asn Ala Pro Val Thr Cys Cys Tyr Asn Phe Thr
 1               5                  10                  15
Asn Arg Lys Ile Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile Thr
            20                  25                  30
Ser Ser Lys Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Ile Val Ala
        35                  40                  45
Lys Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val Gln Asp Ser Met
    50                  55                  60
Asp His Leu Asp Lys Gln Thr Gln Thr Pro Lys Thr
65                  70                  75
```

SEQ ID NO:2

SEQUENCE LENGTH:28

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid terminator

FEATURE

NAME/KEY:terminator

LOCATION:1..28

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION

AGCCCGCCTA ATGAGCGGGC TTTTTTTT 28

SEQ ID NO:3

SEQUENCE LENGTH:18

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— sequence from SD sequence to translation initiation codon

FEATURE

NAME/KEY:RBS

LOCATION:1..8

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION

AAGGAGGTTT AAATTATG 18

SEQ ID NO:4

SEQUENCE LENGTH:850

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— TRP(EB) fragment

FEATURE

NAME/KEY:mat peptide

LOCATION:360..681

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION

AATTCTATCG ACGCAGTGGC ATTTGTCGAG AGTATTCCAT TCTCCGAGAC GCGCGGTTAT 60

GTGAAGAACG TGCTGGCTTA TGACGCTTAC TACCGCTATT TCATGGGGGA TAAACCGACG 120

TTGATGAGCG CCACGGAATG GGGACGTCGT TACTGATCCG CACGTTTATG ATATGCTATC 180

GTACTCTTTA GCGAGTACAA CCGGGGGAGG CATTTTGCTT CCCCCGCTAA CAATGGCGAC 240

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ATATTATG GCC CAA CAA TCA CCC TAT TCA GCA GCG ATG GCA GAA CAG CGT   290
         Ala Gln Gln Ser Pro Tyr Ser Ala Ala Met Ala Glu Gln Arg
           1               5                  10
CAC CAG GAG TGG TTA CGT TTT GTC GAC CTG CTT AAG AAT GCC TAC CAA   338
His Gln Glu Trp Leu Arg Phe Val Asp Leu Leu Lys Asn Ala Tyr Gln
15                  20                  25                  30
AAC GAT CTC CAT TTA CCG TTG TTA AAC CTG ATG CTG ACG CCA GAT GAG   386
Asn Asp Leu His Leu Pro Leu Leu Asn Leu Met Leu Thr Pro Asp Glu
                35                  40                  45
CGC GAA GCG TTG GGG ACT CGC GTG CGT ATT GTC GAA GAG CTG TTG CGC   434
Arg Glu Ala Leu Gly Thr Arg Val Arg Ile Val Glu Glu Leu Leu Arg
        50                  55                  60
GGC GAA ATG AGC CAG CGT GAG TTA AAA AAT GAA CTC GGC GCA GGC ATC   482
Gly Glu Met Ser Gln Arg Glu Leu Lys Asn Glu Leu Gly Ala Gly Ile
        65                  70                  75
GCG ACG ATT ACG CGT GGA TCT AAC AGC CTG AAA GCC GCG CCC GTC GAG   530
Ala Thr Ile Thr Arg Gly Ser Asn Ser Leu Lys Ala Ala Pro Val Glu
    80                  85                  90
CTG CGC CAG TGG CTG GAA GAG GTG TTG CTG AAA AGC GAT TGATTTTGTA    579
Leu Arg Gln Trp Leu Glu Glu Val Leu Leu Lys Ser Asp
 95                 100                 105

GGCCTGATAA GACGTGGCGC ATCAGGCATC GTGCACCGAA TGCCGGATGC GGCGTGAACG  639

CCTTATCCGT CCTACAAATA CCCGTAATTT CAATATGTTT GGTAGGCATG ATAAGACGCG  699

GCAGCGTCGC ATCAGGCGCT TAATACACGG CATTATGAAA CGGACTCAGC GCCAGGATCA  759

CCGCCTGGTG ATAGACGCTG GCGCGAGTGA GTTTCCCGGC GGTAAACACG CCGATCGCCC  819

CTTCCTTACG ACCGATCTCA TCAATAGGAT C                                  850
```

SEQ ID NO:5

SEQUENCE LENGTH:554

SEQUENCE TYPE:nucleic acid

STRANDEDNESS: double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— MCF(SS) fragment

FEATURE

NAME/KEY:mat peptide

LOCATION:40..267

IDENTIFICATION METHOD:S

NAME/KEY:RBS

LOCATION:22..29

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION

```
CTAGTACGCA AGTTCACGTA AAAGGAGGTT TAAATTATG CAG CCA GAT GCA ATC      54
                                          Gln Pro Asp Ala Ile
                                            1               5

AAT GCC CCA GTC ACC TGC TGT TAT AAC TTC ACC AAT AGG AAG ATC TCA    102
Asn Ala Pro Val Thr Cys Cys Tyr Asn Phe Thr Asn Arg Lys Ile Ser
                10                  15                  20

GTG CAG AGG CTC GCG AGC TAT AGA AGA ATC ACC AGC AGC AAG TGT CCC    150
Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile Thr Ser Ser Lys Cys Pro
            25                  30                  35

AAA GAA GCT GTG ATC TTC AAG ACC ATT GTG GCC AAG GAG ATC TGT GCT    198
Lys Glu Ala Val Ile Phe Lys Thr Ile Val Ala Lys Glu Ile Cys Ala
        40                  45                  50

GAC CCC AAG CAG AAG TGG GTT CAG GAT TCC ATG GAC CAC CTG GAC AAG    246
Asp Pro Lys Gln Lys Trp Val Gln Asp Ser Met Asp His Leu Asp Lys
    55                  60                  65

CAA ACC CAA ACT CCG AAG ACT TGATGACTCG AGATCCTCTA CGCCGGACGC       297
Gln Thr Gln Thr Pro Lys Thr
70                  75

ATCGTGGCCG GCATCACCGG CGCCACAGGT GCGGTTGCTG GCGCCTATAT CGCCGACATC  357

ACCGATGGGG AAGATCGGGC TCGCCACTTC GGGCTCATGA GCGCTTGTTT CGGCGTGGGT  417

ATGGTGGCAG GCCCCGTGGC CGGGGGACTG TTGGGCGCCA TCTCCTTGCA TGCACCATTC  477

CTTGCGGCGG CGGTGCTCAA CGGCCTCAAC CTACTACTGG GCTGCTTCCT AATGCAGGAG  537

TCGCATAAGG GAGAGCG                                                 554
```

SEQ ID NO:6

SEQUENCE LENGTH:17

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:othernucleic acid —— chemically synthesized mutagenic primer

SEQUENCE DESCRIPTION

TGACTCGAGG ATCCTCT 17

SEQ ID NO:7

SEQUENCE LENGTH:524

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:othernucleic acid ——— MCF(DS) fragment

FEATURE

NAME/KEY:mat peptide

LOCATION:9..236

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION

```
AAATTATG CAG CCA GAT GCA ATC AAT GCC CCA GTC ACC TGC TGT TAT AAC     50
         Gln Pro Asp Ala Ile Asn Ala Pro Val Thr Cys Cys Tyr Asn
           1               5                  10
TTC ACC AAT AGG AAG ATC TCA GTG CAG AGG CTC GCG AGC TAT AGA AGA     98
Phe Thr Asn Arg Lys Ile Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg
 15                  20                  25                  30
ATC ACC AGC AGC AAG TGT CCC AAA GAA GCT GTG ATC TTC AAG ACC ATT    146
Ile Thr Ser Ser Lys Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Ile
                35                  40                  45
GTG GCC AAG GAG ATC TGT GCT GAC CCC AAG CAG AAG TGG GTT CAG GAT    194
Val Ala Lys Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val Gln Asp
            50                  55                  60
TCC ATG GAC CAC CTG GAC AAG CAA ACC CAA ACT CCG AAG ACT TGATGACTCG 246
Ser Met Asp His Leu Asp Lys Gln Thr Gln Thr Pro Lys Thr
        65                  70                  75

AGGATCCTCT ACGCCGGACG CATCGTGGCC GGCATCACCG GCGCCACAGG TGCGGTTGCT  306

GGCGCCTATA TCGCCGACAT CACCGATGGG GAAGATCGGG CTCGCCACTT CGGGCTCATG  366

AGCGCTTGTT TCGGCGTGGG TATGGTGGCA GGCCCCGTGG CCGGGGGACT GTTGGGCGCC  426

ATCTCCTTGC ATGCACCATT CCTTGCGGCG GCGGTGCTCA ACGGCCTCAA CCTACTACTG  486

GGCTGCTTCC TAATGCAGGA GTCGCATAAG GGAGAGCG                          524
```

SEQ ID NO:8

SEQUENCE LENGTH:46

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized DNA

SEQUENCE DESCRIPTION

TCGAGTAGCC CGCCTAATGA GCGGGCTTTT TTTTCGCTTG AATTCG 46

SEQ ID NO:9

SEQUENCE LENGTH:46

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized DNA

ANTI-SENSE:Yes

SEQUENCE DESCRIPTION

GATCCGAATT CAAGCGAAAA AAAAGCCCGC TCATTAGGCG GGCTAC 46

SEQ ID NO:10

SEQUENCE LENGTH:50

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized DNA

OTHER INFORMATION:there exists a base sequence of "CTAG" in the complementary strand as a base sequence No. 47-50 in addition to a complementary strand corresponding to a base sequence No. 5-46.

SEQUENCE DESCRIPTION

TCGAGTAGCC CGCCTAATGA GCGGGCTTTT TTTTCGCTTG AATTCG 46

SEQ ID NO:11

SEQUENCE LENGTH:30

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:othernucleic acid —— chemically synthesized mutagenic primer

SEQUENCE DESCRIPTION

GAGGTTTAAA TTATGCCAGA TGCAATCAAT 30

SEQ ID NO:12

SEQUENCE LENGTH:30

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized mutagenic primer

SEQUENCE DESCRIPTION

GAGGTTTAAA TTATGGCAAT CAATGCCCCA 30

SEQ ID NO:13

SEQUENCE LENGTH:30

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized mutagenic primer

SEQUENCE DESCRIPTION

GAGGTTTAAA TTATGGCCCC AGTCACCTGC 30

SEQ ID NO:14

SEQUENCE LENGTH:14

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized DNA

SEQUENCE DESCRIPTION

CAACAATCAC CCTA 14

SEQ ID NO:15

SEQUENCE LENGTH:979

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:chromosome DNA

ORIGINAL SOURCE:

TISSUE TYPE:E. coli K-12 strain

CELL LINE:HB101

FEATURE

NAME/KEY:CDS

LOCATION:356..683

IDENTIFICATION METHOD:S

NAME/KEY:mat peptide

LOCATION:360..681

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION

```
ACCAGTTACC TGCAATATGT TTATCAGCAG TTTGGCAATA ATCGTATTTT CTCCTCAGCA   60

GCTTATAACG CCGGACTAGG GCGGGTGCGA ACCTGGCTTG GCAACAGCGC CGGGCGTATC  120

GACGCAGTGG CATTTGTCGA GAGTATTCCA TTCTCCGAGA CGCGCGGTTA TGTGAAGAAC  180

GTGCTGGCTT ATGACGCTTA CTACCGCTAT TTCATGGGGG ATAAACCGAC GTTGATGAGC  240

GCCACGGAAT GGGGACGTCG TTACTGATCC GCACGTTTAT GATATGCTAT CGTACTCTTT  300

AGCGAGTACA ACCGGGGGAG GCATTTTGCT TCCCCCGCTA ACAATGGCGA CATATTATG   359

GCC CAA CAA TCA CCC TAT TCA GCA GCG ATG GCA GAA CAG CGT CAC CAG      407
Ala Gln Gln Ser Pro Tyr Ser Ala Ala Met Ala Glu Gln Arg His Gln
 1               5                  10                  15

GAG TGG TTA CGT TTT GTC GAC CTG CTT AAG AAT GCC TAC CAA AAC GAT     455
Glu Trp Leu Arg Phe Val Asp Leu Leu Lys Asn Ala Tyr Gln Asn Asp
            20                  25                  30

CTC CAT TTA CCG TTG TTA AAC CTG ATG CTG ACG CCA GAT GAG CGC GAA     503
Leu His Leu Pro Leu Leu Asn Leu Met Leu Thr Pro Asp Glu Arg Glu
        35                  40                  45

GCG TTG GGG ACT CGC GTG CGT ATT GTC GAA GAG CTG TTG CGC GGC GAA     551
Ala Leu Gly Thr Arg Val Arg Ile Val Glu Glu Leu Leu Arg Gly Glu
    50                  55                  60

ATG AGC CAG CGT GAG TTA AAA AAT GAA CTC GGC GCA GGC ATC GCG ACG     599
Met Ser Gln Arg Glu Leu Lys Asn Glu Leu Gly Ala Gly Ile Ala Thr
65                  70                  75                  80

ATT ACG CGT GGA TCT AAC AGC CTG AAA GCC GCG CCC GTC GAG CTG CGC     647
Ile Thr Arg Gly Ser Asn Ser Leu Lys Ala Ala Pro Val Glu Leu Arg
                85                  90                  95

CAG TGG CTG GAA GAG GTG TTG CTG AAA AGC GAT TGATTTTGTA GGCCTGATAA   700
Gln Trp Leu Glu Glu Val Leu Leu Lys Ser Asp
            100                 105

GACGTGGCGC ATCAGGCATC GTGCACCGAA TGCCGGATGC GGCGTGAACG CCTTATCCGT   760

CCTACAAATA CCCGTAATTT CAATATGTTT GGTAGGCATG ATAAGACGCG GCAGCGTCGC   820

ATCAGGCGCT TAATACACGG CATTATGAAA CGGACTCAGC GCCAGGATCA CCGCCTGGTG   880

ATAGACGCTG GCGCGAGTGA GTTTCCCGGC GGTAAACACG CCGATCGCCC CTTCCTTACG   940

ACCGATCTCA TCAATACCGG TATAACGCGA CATCACGGG                          979
```

SEQ ID NO:16

SEQUENCE LENGTH:30

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized mutagenic primer

SEQUENCE DESCRIPTION

CAGCGCCGGG CGGAATTCTA TCGACGCAGT 30

SEQ ID NO:17

SEQUENCE LENGTH:30

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized mutagenic primer

SEQUENCE DESCRIPTION

ATCTCATCAA TAGGATCCCC GGTATAACGC 30

SEQ ID NO:18

SEQUENCE LENGTH:741

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:cDNA to mRNA

ORIGINAL SOURCE:

TISSUE TYPE:human promyelocytic leukaemia cells

CELL LINE:HL-60

FEATURE

NAME/KEY:CDS

LOCATION:70..369

IDENTIFICATION METHOD:S

NAME/KEY:sig peptide

LOCATION:70..138

IDENTIFICATION METHOD:S

NAME/KEY:mat peptide

LOCATION:139..366

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION

```
AACCGAGAGG CTGAGACTAA CCCAGAAACA TCCAATTCTC AAACTGAAGC TCGCACTCTC   60

GCCTCCAGC ATG AAA GTC TCT GCC GCC CTT CTG TGC CTG CTG CTC ATA GCA  111
          Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Ile Ala
                      -20                 -15                 -10
GCC ACC TTC ATT CCC CAA GGG CTC GCT CAG CCA GAT GCA ATC AAT GCC    159
Ala Thr Phe Ile Pro Gln Gly Leu Ala Gln Pro Asp Ala Ile Asn Ala
                  -5                  1               5
CCA GTC ACC TGC TGT TAT AAC TTC ACC AAT AGG AAG ATC TCA GTG CAG    207
Pro Val Thr Cys Cys Tyr Asn Phe Thr Asn Arg Lys Ile Ser Val Gln
        10                  15                  20
AGG CTC GCG AGC TAT AGA AGA ATC ACC AGC AGC AAG TGT CCC AAA GAA    255
Arg Leu Ala Ser Tyr Arg Arg Ile Thr Ser Ser Lys Cys Pro Lys Glu
    25                  30                  35
GCT GTG ATC TTC AAG ACC ATT GTG GCC AAG GAG ATC TGT GCT GAC CCC    303
Ala Val Ile Phe Lys Thr Ile Val Ala Lys Glu Ile Cys Ala Asp Pro
 40                  45                  50                  55
AAG CAG AAG TGG GTT CAG GAT TCC ATG GAC CAC CTG GAC AAG CAA ACC    351
Lys Gln Lys Trp Val Gln Asp Ser Met Asp His Leu Asp Lys Gln Thr
                60                  65                  70
CAA ACT CCG AAG ACT TGAACACTCA CTCCACAACC CAAGAATCTG CAGCTAACTT    406
Gln Thr Pro Lys Thr
            75

ATTTTCCCCT AGCTTTCCCC AGACACCCTG TTTTATTTTA TTATAATGAA TTTTGTTTGT  466

TGATGTGAAA CATTATGCCT TAAGTAATGT TAATTCTTAT TTAAGTTATT GATGTTTTAA  526

GTTTATCTTT CATGGTACTA GTGTTTTTTA GATACAGAGA CTTGGGGAAA TTGCTTTTCC  586
```

TCTTGAACCA CAGTTCTACC CCTGGGATGT TTTGAGGGTC TTTGCAAGAA TCATTAATAC 646

AAAGAATTTT TTTTAACATT CCAATGCATT GCTAAAATAT TATTGTGGAA ATGAATATTT 706

TGTAACTATT ACACCAAATA AATATATTTT TGTAC 741

SEQ ID NO:19

SEQUENCE LENGTH:34

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized mutagenic primer

SEQUENCE DESCRIPTION

CAAGGGCTCG CTTTTAAATT ATGCAGCCAG ATGC 34

SEQ ID NO:20

SEQUENCE LENGTH:34

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— chemically synthesized mutagenic primer

SEQUENCE DESCRIPTION

CCGAAGACTT GATGACTCGA GACACTCACT CCAC 34

SEQ ID NO:21

SEQUENCE LENGTH:11

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— inserted DNA

SEQUENCE DESCRIPTION

TTTAAATTAT G 11

SEQ ID NO:22

SEQUENCE LENGTH:418

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— (DraI)(XhoI) inserted phage DNA

FEATURE

NAME/KEY:sig peptide

LOCATION:70..138

IDENTIFICATION METHOD:S

NAME/KEY:mat peptide

LOCATION:150..377

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION

```
AACCGAGAGG CTGAGACTAA CCCAGAAACA TCCAATTCTC AAACTGAAGC TCGCACTCTC   60

GCCTCCAGC ATG AAA GTC TCT GCC GCC CTT CTG TGC CTG CTG CTC ATA GCA  111
          Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Ile Ala
                     -20                 -15                 -10
GCC ACC TTC ATT CCC CAA GGG CTC GCT TTTAAATTAT G CAG CCA GAT GCA   161
Ala Thr Phe Ile Pro Gln Gly Leu Ala              Gln Pro Asp Ala
                  -5                                  1
ATC AAT GCC CCA GTC ACC TGC TGT TAT AAC TTC ACC AAT AGG AAG ATC    209
Ile Asn Ala Pro Val Thr Cys Cys Tyr Asn Phe Thr Asn Arg Lys Ile
  5                 10                  15                  20
TCA GTG CAG AGG CTC GCG AGC TAT AGA AGA ATC ACC AGC AGC AAG TGT    257
Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile Thr Ser Ser Lys Cys
                  25                  30                  35
CCC AAA GAA GCT GTG ATC TTC AAG ACC ATT GTG GCC AAG GAG ATC TGT    305
Pro Lys Glu Ala Val Ile Phe Lys Thr Ile Val Ala Lys Glu Ile Cys
             40                  45                  50
```

GCT GAC CCC AAG CAG AAG TGG GTT CAG GAT TCC ATG GAC CAC CTG GAC 353
Ala Asp Pro Lys Gln Lys Trp Val Gln Asp Ser Met Asp His Leu Asp
55 60 65

AAG CAA ACC CAA ACT CCG AAG ACT TGATGACTCG AGACACTCAC TCCACAACCC 407

Lys Gln Thr Gln Thr Pro Lys Thr
70 75

AAGAATCTGC A 418

SEQ ID NO:23

SEQUENCE LENGTH:337

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:double

TOPOLOGY:linear

MOLECULE TYPE:other nucleic acid —— E.coli trp-SD sequence

FEATURE

NAME/KEY:-35 signal

LOCATION:280..285

IDENTIFICATION METHOD:S

NAME/KEY:-10 signal

LOCATION:303..308

IDENTIFICATION METHOD:S

NAME/KEY:RBS

LOCATION:328..335

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION

AATTCCATTC AACGCCAGTC CCGAACGTGA AATTTCCTCT CTTGCTGGCG CGATTGCAGC 60

TGTGGTGTCA TGGTCGGTGA TCGCCAGGGT GCCGACGCGC ATCTCGACTG CACGGTGCAC 120

CAATGCTTCT GGCGTCAGGC AGCCATCGGA AGCTGTGGTA TGGCTGTGCA GGTCGTAAAT 180

CACTGCATAA TTCGTGTCGC TCAAGGCGCA CTCCCGTTCT GGATAATGTT TTTTGCGCCG 240

ACATCATAAC GGTTCTGGCA AATATTCTGA AATGAGCTGT TGACAATTAA TCATCGAACT 300

AGTTAACTAG TACGCAAGTT CACGTAAAAG GAGGTTT 337

CLAIMS

1. A process for producing a polypeptide having monocyte chemotactic activity which comprises:

using an expression plasmid

having a translation termination codon and a terminator sequence downstream of the structural gene and

a translation initiation codon,

a ribosome binding site sequence and

a promotor sequence derived from E. coli

connected upstream of the structural gene; and

using as a host E. coli

an E. coli LE392 strain, an E. coli BL21(DE3) strain,

an E. coli AB1899 strain, an E. coli B/r-WP2-hcr$^-$ strain or

an E. coli C600 hflA strain, or

a mutant strain thereof;

in the production of a polypeptide having monocyte chemotactic activity as an unfused polypeptide by constructing an expression plasmid in which there has integrated DNA which encodes a polypeptide having monocyte chemotactic activity consisting of an amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence which has truncated N-terminal 1-6 amino acids thereof,

transforming E. coli as a host by introducing said expression plasmid thereinto, and

cultivating the transformed E. coli.

2. A process for producing a polypeptide having monocyte chemotactic activity which comprises:

using an expression plasmid:

having a translation termination codon and a terminator

sequence downstream of the structural gene

and optionally a repressor gene derived from E. coli

inserted and

a translation initiation codon,

a ribosome binding site sequence and

a promotor sequence derived from E. coli

connected upstream of the structural gene;

and using as a host E. coli:

an E. coli LE392 strain, an E. coli BL21(DE3) strain,

an E. coli AB1899 strain, an E. coli B/r-WP2-hcr$^-$ strain or

an E. coli C600 hflA strain, or

a mutant strain thereof;

in the production of a polypeptide having monocyte chemotactic activity as an unfused polypeptide by constructing an expression plasmid in which there has integrated DNA which encodes a polypeptide having monocyte chemotactic activity consisting of an amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence which has truncated N-terminal 1-6 amino acids thereof,

transforming E. coli as a host by introducing said expression plasmid thereinto, and

cultivating the transformed E. coli.

3. The process of claim 2, in which the terminator sequence is a base sequence represented by SEQ ID NO:2.

4. The process of claim 2, in which the terminator sequence is a base sequence represented by SEQ ID NO:2 and the sequence from the translation initiation codon to the ribosome binding site sequence is a base sequence represented by SEQ ID NO:3.

5. The process of claim 2, in which the mutant strain of host E. coli is an antibiotic-resistant strain, thymine-requiring strain, recA

gene mutant strain or lysogenic strain for bacteriophage of an E. coli LE392 strain, an E. coli BL21(DE3) strain, an E. coli AB1899 strain, an E. coli B/r-WP2-hcr⁻ strain or an E. coli C600 hflA strain.

6. The process of claim 2, in which the repressor gene is a base sequence represented by SEQ ID NO:4, the promotor sequence is a tryptophan promotor operator derived from E. coli, and E. coli as a host is an E. coli LE392 strain or a mutant strain thereof.

7. The process of claim 2, in which the mutant strain of host E. coli is a mutant strain of an E. coli LE392 strain, an E. coli BL21(DE3) strain, an E. coli AB1899 strain, an E. coli B/r-WP2-hcr⁻ strain or an E. coli C600 hflA strain which has at least two properties selected from antibiotic-resistance, thymine-requirement, mutation of recA gene and lysogenesis for bacteriophage.

8. The process of claim 2, in which the host E. coli is an E. coli LE392 strain or a strain thereof conferring rifamycin-resistant and recA gene mutation.

9. The process of claim 2, in which the terminator sequence is a base sequence represented by SEQ ID NO:2; the sequence from the translation initiation codon to the ribosome binding site sequence is a base sequence represented by SEQ ID NO:3; the repressor gene is a base sequence represented by SEQ ID NO:4; the promotor sequence is a tryptophan promotor operator derived from E. coli; and E. coli as a host is a mutant strain of the E. coli LE392 strain conferring rifamycin-resistant and recA gene mutation.

10. An E. coli LE392 strain, an E. coli BL21(DE3) strain, an E. coli AB1899 strain, an E. coli B/r-WP2-hcr⁻ strain or an E. coli C600 hflA strain, or a mutant strain thereof, which is transformed with an expression plasmid having a translation termination codon and a terminator sequence downstream and a translation initiation codon, a

ribosome binding site sequence and a promotor sequence derived from E. coli connected upstream of DNA encoding a polypeptide having monocyte chemotactic activity which consists of an amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence which has truncated N-terminal 1 to 6 amino acids thereof.

11. An E. coli LE392 strain, an E. coli BL21(DE3) strain, an E. coli AB1899 strain, an E. coli B/r-WP2-hcr$^-$ strain or an E. coli C600 hflA strain, or a mutant strain thereof, which is transformed with an expression plasmid having a translation termination codon and a terminator sequence downstream and optionally a repressor gene derived from E. coli inserted, and a translation initiation codon, a ribosome binding site sequence and a promotor sequence derived from E. coli connected upstream of DNA encoding a polypeptide having monocyte chemotactic activity which consists of an amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence which has truncated N-terminal 1 to 6 amino acids thereof.

12. The E. coli strain or its mutant strain of claim 11, which is transformed with an expression plasmid having a base sequence represented by SEQ ID NO:2 as a terminator sequence.

13. The E. coli strain or its mutant strain of claim 11 which is transformed with an expression plasmid having a base sequence represented by SEQ ID NO:2 as a terminator sequence and a base sequence represented by SEQ ID NO:3 as a sequence from the translation initiation codon to the ribosome binding site sequence.

14. The E. coli strain or its mutant strain of claim 11, in which the transformed E. coli is an E. coli LE392 strain, or a strain thereof conferring rifamycin resistant and recA gene mutation.

15. The mutant strain of an E. coli LE392 strain conferring rifamycin resistant and recA gene mutation of claim 11, which is

transformed with an expression plasmid having a base sequence represented by SEQ ID NO:2 as a terminator sequence; a base sequence represented by SEQ ID NO:3 as a sequence from the translation initiation codon to the ribosome binding site sequence; a base sequence represented by SEQ ID NO:4 as a repressor gene sequence; and a tryptophan promotor operator derived from E. coli.

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP 92/00550

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/19; C12N15/71; C12N1/21; //(C12N1/21 C12R1:19)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; C12R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	WO,A,9 007 863 (UNITED STATES OF AMERICA) 26 July 1990 cited in the application see the whole document , especially page 3 line 33 to page 4 line 36, examples 2-4 and the claims	1-15
Y	JOURNAL OF BIOCHEMISTRY. vol. 106, 1989, TOKYO JP pages 436 - 441; RYUJI FURUTA ET AL: 'Production and characterization of recombinant human neutrophil chemotactic factor' cited in the application see page 437	1-15

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
03 AUGUST 1992	12. 08. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LE CORNEC N.D.R.

2

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. vol. 159, no. 1, 28 February 1989, DULUTH, MINNESOTA US pages 249 - 255; YASUJI FURUTANI ET AL: 'Cloning and sequencing of the cDNA for human monocyte chemotactic and activating factor;MCAF' cited in the application	1
Y	GHERNA R., PIENTA P. & COTE R. 'ATCC , CATALOGUE OF BACTERIA AND PHAGES .17th edition' 1989 , ATCC , ROCKVILLE, US see page 86 nO 23231 and 23233 and page 91 nO 33572	1-15
Y	JOURNAL OF MOLECULAR BIOLOGY. vol. 189, no. 1, 5 May 1986, LONDON ;GB. pages 113 - 130; F. W. STUDIER ET AL: 'Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes' cited in the application see the whole document	1-15
A	NUCLEIC ACIDS RESEARCH. vol. 9, 1981, ARLINGTON, VIRGINIA US pages 6647 - 6667; C. YANOFSKY AT AL: 'The complete nucleotide sequence of the tryptophan operon of E. coli' see the whole document	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. JP 9200550 SA 58826

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 03/08/92
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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